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The estrogen receptor a is transcription factor that is regulated by ligand binding and phosphorylation. The phosphorylation of three serine residues (S104, S106 and S118) located within the N-terminal transcriptional activation domain (AF-1) of the receptor are important for full receptor transcriptional activation. We provide evidence that alteration in the regulation of cyclinA/Cdk2 complex lead to changes in hormone-dependent and hormone-independent transcriptional enhancement by ER. This is accomplished, at least in part, through the direct phosphorylation of ER S104, S106 and S118 by the cyclinA/Cdk2 complex. Since alterations in cyclin dependent kinase regulatory proteins are a common feature in breast cancer, these findings provide a framework for understanding the mechanism by which CDK dysregulation can directly affect ER signaling through alterations in receptor phosphorylation.

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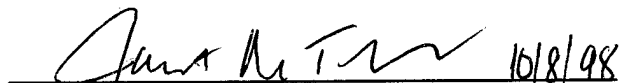

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TABLE OF CONTENTS

5. INTRODUCTION

The estrogen receptor (ER) is a ligand-dependent transcriptional regulatory protein that controls the genetic programs affecting many aspects of cell growth and differentiation. In addition to ligand binding, phosphorylation plays an important role in regulating ER function. The receptor contains sites for both constitutive and ligand-dependent phosphorylation (2, 13, 28). Three serine residues (amino acids 104, 106 and 118) located within the N-terminal activation domain (AF-1) and one residue in the hinge region, S294, match the consensus sequence recognized by a family of ser/thr pro-directed kinases that include cyclin-dependent kinases (CDK), mitogen-activated protein kinases (MAPK) and glycogen synthase kinase-3 (GSK-3). Serines 104, 106, and 118 are phosphorylated upon hormone treatment; serine to alanine mutations at these positions decrease ligand-dependent transcriptional activity (2, 25, 28). Accumulating evidence suggests that MAPK can phosphorylate Ser 118 and that this may lead to estradiol-independent ER activation or, alternatively, an increase in ligand-dependent ER activation (3, 9, 24). However, whether cyclin-dependent kinases target ER as a substrate for phosphorylation and affect its transcriptional activity remains unclear. Recently, a CDK-independent effect of cyclin D1 upon ER-dependent transcriptional activity was reported in T-47D breast cancer cells(43) A link between cyclin-dependent kinase enhancement of ER function and attendent receptor phosphorylation has not yet been demonstrated.

Cyclin-dependent kinases are a family of proteins composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. The cyclin subunit appears to regulate subcellular localization, timing of activation as well as substrate specificity of the kinase complex. Cyclin-CDK complexes regulate the activity of target molecules, including transcriptional regulatory proteins, by phosphorylation. Regulation of cyclin-CDK activity is accomplished by proteins that activate (CDK activators or CAKs), or inhibit (CDK inhibitors or CDIs), kinase function (16, 17, 30, 31). Because cyclin-dependent kinases control cell division, the dysregulation of cyclins, their kinase partners, and/or the upstream regulatory CAKs and CDIs, have been implicated in the initiation and promotion of hyperplasia and oncogenesis. In fact, the overexpression of the regulatory cyclin subunit and the dysregulation of the catalytic CDK subunit have been identified in a number of solid tumors, leukemias, and tumor-derived cell lines (6, 8, 10-12, 15, 18, 19, 26, 29, 40, 43).

This study examines the effects of the cyclin A/CDK2 complex on ER transcriptional activation. We chose to focus on cyclin A for several reasons: (1) Cyclin A plays a multifaceted role in cell cycle progression and is a key regulator of CDK2, a ser/thr pro-directed kinase with the potential to phosphorylate ER. (2) Cyclin A expression is cell

adhesion-dependent. Cyclin A overexpression can lead to adhesion-independent cell growth, a hallmark of cellular transformation (5, 20). (3) The synthesis and degradation of cyclin A are tightly regulated, suggesting that its aberrant expression could seriously jeopardize the control of cell growth (22, 34). (4) Cyclin A overexpression has been implicated as an important indicator of oncogenesis in several contexts including human breast tumor-derived cell lines and a mouse mammary tumor virus breast cancer model (7, 8, 15, 21, 35, 42). (5) Cyclin A shares several features with the proto-oncogene cyclin D1 including the ability to bind to and phosphorylate Rb, such that inappropriate cyclin A expression leads to perturbations in the regulation of the G1 to S transition (33). (6) Recent reports have also linked the degradation of $p27^{KIP1}$ (hereafter referred to as p27), an inhibitor of cyclin A/CDK2 kinase activity, and aggressive breast and colorectal cancers (10, 29, 40). Together, these findings suggest that cyclin A may function as a proto-oncogene.

To determine whether the cyclin A/CDK2 complex can affect ER function, we have examined the consequences of activation or inhibition of the cyclin A/CDK2 pathway on ER-dependent transcriptional activation. We find that ectopic expression of cyclin A or activation of the endogenous cyclin A/CDK2 complex increases ER-dependent transcriptional enhancement, whereas inhibition of cyclin A/CDK2 activity decreases receptor-dependent transcriptional activation. In addition, we have examined whether the cyclin A/CDK2 kinase complex has the capacity to phosphorylate ER and have found that the cyclin A/CDK2 complex phosphorylates ER *in vivo* and *in vitro*. This interaction between ER and a cyclin-CDK complex links receptor-dependent transcriptional regulation with cell growth control through direct receptor phosphorylation.

6. BODY

Increased ER transcriptional enhancement by ectopic cyclin A expression.

To establish whether ectopic expression of cyclin A affects estrogen receptor-dependent activation, we examined the ability of cyclin A to increase ER-mediated transcriptional enhancement. Estrogen receptor-deficient HeLa cells were transfected with an expression vector for the full length human ER containing a FLAG epitope at its N-terminus, the reporter plasmid ERE-tk-CAT, plasmids encoding human cyclin A and a constitutive b-galactosidase expression vector as an internal transfection standard. Transfected cells were treated with 17b-estradiol or the ethanol vehicle for 24 hours. Transcriptional activity was measured by CAT assay and normalized to b-galactosidase activity. As shown in Figure 1A, both hormone-dependent and hormone-independent ER

transcriptional activity was increased roughly 3-fold when cyclin A is overexpressed. No effect of cyclin A on reporter gene activity was observed in the absence of ER (data not shown). To ensure that this increased transcriptional activity was not a result of additional ER protein production, we monitored protein expression in whole cell extracts using Western blot analysis. Figure 1B illustrates ER levels are not increased by cyclin A co-expression (compare lanes 5-6 to lanes 7-8). In addition, cyclin A is expressed above endogenous levels as a result of our transient transfection scheme and estradiol treatment does not alter cyclin A expression (Fig. 1B, compare lanes 1-2 to lanes 3-4). By increasing the amount of cyclin A used in these transfections, we were able to observe a concomitant increase in ER transcriptional activation (Fig. 1C). Co-expression of cyclin A and CDK2 also results in an increased ER-dependent transcriptional activity slightly above that of cyclin A alone. Expression of CDK2 alone, on the other hand, did not significantly alter the ER-dependent transcriptional activity (data not shown). These findings suggests that cyclin A is a limiting factor for full hormone-dependent ER-mediated transcriptional enhancement, presumably by favoring the formation of active cyclin-CDK complexes from endogenous CDK2 subunits. Thus, cyclin A expression greatly magnifies the characteristic hormone-dependent ER transcriptional response, which suggests that this cyclin-CDK complex can act as an effector of the estrogen receptor signaling pathway.

Reciprocal effects of cyclin-CDK activators and inhibitors on ER transcriptional enhancement.

To further demonstrate that alterations in cyclin A/CDK2 kinase activity can modify ER activity, we used two classes of CDK regulatory proteins, CAKs, which are composed of cyclin H and CDK7, and the CDI, p27. p27 inhibits many cyclin-CDK complexes, including cyclin A/CDK2, cyclin E/CDK2, and cyclin D2/CDK4 (32, 41). We were particularly interested in studying p27 in light of recent reports linking its premature or excessive degradation to aggressive breast and colorectal cancers (10, 29, 40).

Figure 2A illustrates that CDK activation by expression of cyclin A/CDK2 or CAK (cyclin H and CDK7) leads to a 2-fold increase in ER transcriptional enhancement. The co-expression of all four proteins, the cyclin-CDK complex as well as the CAK complex, further augments (4-fold) this response and lends further support for cyclin-CDK involvement in the regulation of ER-dependent transcriptional activity.

We next asked if a decrease in cyclin-CDK kinase activity would reduce estrogen receptor-dependent transcriptional activation. We chose two means of inhibiting CDK2 kinase activity. Initially, the cyclin-dependent kinase inhibitor, p27, was ectopically expressed in HeLa cells and ER-dependent transcriptional enhancement was measured.

Ligand-dependent transcriptional activation by ER was greatly reduced by p27 expression (Fig. 2B). This effect is noted in either the presence or absence of ectopically expressed cyclin A. Therefore, reducing CDK activity leads to impaired ER transcriptional activity.

At this point in our studies, we could not discriminate between an effect of p27 upon cdc2, CDK2, or CDK4, since p27 can inhibit all of these kinases. Therefore, we sought another means of reducing CDK2 activity by using a catalytically inactive CDK2 mutant to specifically block endogenous CDK2 activity. This CDK derivative, designated CDK2TS, is competent for cyclin A binding, but it cannot bind to ATP due to two consecutive amino acid changes in the ATP-binding site (lysines 33 and 34 are replaced by threonine and serine, respectively). This mutant acts as a dominant negative by sequestering cyclin A, thereby preventing it from binding and activating endogenous wild type CDK2.

By expressing the dominant negative CDK2 mutant, we were able to reduce significantly the ER response to ligand treatment (Fig. 2B). Ectopic expression of a dominant negative cdc2 mutant had little effect on ER activity (data not shown). These results strongly argue that the observed decrease of ER transcriptional activity by p27 is due to inactivation of CDK2 and further suggests the importance of cyclin A/CDK2 kinase activity for hormone-dependent transcriptional enhancement by ER. It appears then, that the *balance* between the CDK regulatory proteins, CAKs and CDIs, is a critical step in determining ER transcriptional activity.

Increased ER Transcriptional Enhancement in Response to Ectopic Cyclin A Expression Occurs in Multiple Cell Lines

In order to test our hypothesis that the regulation of ER-dependent transcriptional activity by the cyclin A/CDK2 complex is not specific to HeLa cells, but rather reflects a general mode of regulation, we repeated our transcriptional activity assay in a variety of cell lines. We tested Hs 578Bst cells derived from breast tissue peripheral to an infiltrating ductal carcinoma, U-2 OS human osteosarcoma cells and a cell line derived from a pleural effusion of a mammary adenocarcinoma, SK-BR-3. All three human cell lines are ER-negative. Cells were transiently transfected with plasmids encoding wtER and cyclin A as described previously, treated with 17b-estradiol or the ethanol vehicle for 24 hours and transcriptional activity was measured. In the three cell types utilized, we observed a significant increase in ER ligand-dependent transcriptional activation (Figure 3, B, C and D). There was also an increase in ligand-independent activity as has been observed for HeLa cells, although the magnitude of the effect varied from line to line. These data imply

that the ability of a cyclin A/CDK2 complex to enhance ER ligand-dependent transcription is conserved across multiple cell types.

Phosphorylation of ER by the cyclin A/CDK2 kinase complex.

Next, we investigated whether the cyclin A/CDK2 complex can phosphorylate the estrogen receptor protein. To determine if ectopic expression of cyclin A increased the amount of phosphate incorporated into ER *in vivo,* HeLa cells were transfected with ER alone or in combination with cyclin A and cells were metabolically labeled with ^{32}P-orthophosphate for two hours in the presence or absence of 17b-estradiol. For each sample, the total amount of ER visualized by silver staining was used to standardize the amount of incorporated ^{32}P. The untreated ER condition was arbitrarily set as 1. As shown in Fig. 4A, ER phosphorylation is increased by ectopic expression of cyclin A in both the absence (3x) and presence (3.7x) of hormone. Thus, the presence of cyclin A increases incorporation of phosphate into ER by activating endogenous CDKs.

In order to further investigate the effect of cyclin A/CDK2-dependent phosphorylation of ER we performed *in vitro* kinase assays. Three ER derivatives, containing amino acids 1-82, 1-115 or 1-121 were bacterially expressed and purified as GST-fusion proteins and used as substrates for phosphorylation by immunopurified baculovirus-expressed cyclin A and CDK2. These particular derivatives were chosen since the ER 1-121 derivative contains three ser/thr-pro residues, serines 104, 106 and 118, whereas ER 1-115 contains only serines 104 and 106. ER 1-82 lacks all of the putative ser/thr-pro phosphorylation sites and thus serves as a negative control. Figure 4B demonstrates that both ER 1-121 and ER 1-115 were phosphorylated by the cyclin A/CDK2 complex but not with either subunit alone. On the other hand, ER 1-82 was not phosphorylated by the cyclin A/CDK2 complex. In each reaction, expression of the ER substrate and the kinase subunits was verified by Western blotting and found to be identical (data not shown). The fact that ER 1-121 and ER 1-115 derivatives were phosphorylated while ER 1-82 was not, strongly suggests that the residues contained in the region comprised by amino acids 83 through 121 comprise a motif targeted by the cyclin A/CDK2 kinase complex. This data provide *in vitro* biochemical evidence that the estrogen receptor is a substrate for cyclin/CDK-dependent phosphorylation.

ER is Phosphorylated by the Cyclin A/CDK2 Complex at Serine 104, Serine 106 and Serine 118 *in vitro*

Mutants bearing individual serine to threonine changes at either S104 (S104T), S106 (S106T) or S118 (S118T) were cloned into a bacterial expression vector encoding the

first 121 amino acids of ER as a GST fusion protein. The strategy for this amino acid substitution ans subsequent *in vitro* kinase assays is outlined in Figure 5. The fusion proteins were used as substrates for the cyclin A/CDK2 complex in an *in vitro* kinase assay. The reaction products were separated by 12.5% SDS-PAGE, transferred to Immobilon paper, and subjected to either phosphoamino acid analysis or Western blotting using a phosphothreonine-specific monoclonal antibody. Figure 6 shows the results of phosphoamino acid analysis performed on the three serine to threonine mutants (S104T, S106T and S118T) compared to the wtER. wtER is phosphorylated by the cyclin A/CDK2 complex and contains exclusively phosphoserine; no phosphothreonine is detected (WT, Figure 13). In each case, changing a serine to a threonine at a single site (S104T, S106T, S118T) results in the detection of a phosphothreonine following phosphoamino acid analysis, indicating that cyclin A/CDK2 is capable of phosphorylating residues 104, 106 and 118 *in vitro*.

Phosphorylation Site-Specific Mutations Decrease the Effect of Ectopic Cyclin A Expression on ER

In view of our biochemical data we have extended our studies to include *in vivo* assays of transcriptional activation by the phosphorylation site-specific mutants. We have used the single and combination serine to alanine constructs (S104A, S106A, S118A, S104A/S106A, S104A/S118A, S106A/S118A, S104A/S106A/S118A) in transient transfection experiments in U-2 OS cells, which do not contain endogenous ER, to determine the effect of these mutations upon the previously observed cyclin A-dependent enhancement of ER transcriptional activation. The effects of serine to alanine changes are summarized in Figure 7. In each case, the activity of the mutant receptor is compared to that of wild type in the presence or absence of ectopically expressed cyclin A. Changing a serine to an alanine at each position, either single or in combination with serine to alanine mutations at other positions, decreases ER-transcriptional enhancement in response to cyclin A co-transfection. This effect is not a result of a decrease in mutant receptor expression levels, since the expression of all of the phosphorylation site receptor mutants has been confirmed by Western blotting. There is also no effect of the single phosphorylation site mutants upon the transcriptional activity of the receptor in the absence of cyclin A co-transfection (data not shown). This strongly implies that the observed effect of cyclin A enhancement upon ER-transcriptional activity is being mediated through these residues *in vivo*.

Figure 7 and Table 1 provide a summary of data obtained in several experiments comparing the average percentage of wtER activity in the presence of estradiol under

conditions of cyclin A co-transfection. Single serine to alanine mutations at S104, S106 and S118 decrease ER activation by 48%, 32%, and 41%, respectively. The double mutants demonstrate a further decrease in ER activity; S104A/S106A and S106A/S118A are decreased by 54% and 58%, respectively. The most dramatic decreases in receptor activity, 78% and 63%,were observed for the S104A/S118A and S104A/S106A/S118A mutants, respectively.

We conclude from these *in vivo* data that the cyclin A/CDK2 complex increases ER-dependent transcriptional activity by phosphorylating S104, S106 and S118. We have shown biochemically that cyclin A/CDK2 phosphorylates all three residues. We have also demonstrated that these sites are important *in vivo* for mediating the cyclin A/CDK2 enhancement of ER-dependent transcriptional activity. Impairing phosphorylation at individual sites (S104A, S106A and S106A) decreases receptor activity to varying degrees, whereas double mutants (S104A/S106A, S104A/S118A, and S106A/S118A) and the triple mutant (S104A/S106A/S118A) are further compromised in their ability to respond to cyclin A overexpression.

Experimental Materials and Methods

Plasmids and cDNAs

The ΔETCO reporter plasmid contains one estrogen response element (ERE) upstream from the herpes simplex virus thymidine kinase (tk) promoter (-109) driving the expression of the chloramphenicol acetyl transferase (CAT) gene. This reporter lacks a nearby AP-1 binding site to ensure that the results obtained are not influenced by other regulatory elements in the plasmid. The reporter plasmid XETL (also known as ERE$_{tk}$Luc) (9) contains a single ERE from the *Xenopus* vitellogenin A2 gene, the herpes simplex virus tk promoter, and the firefly luciferase coding sequence. This reporter, like ΔETCO, lacks an AP-1 site. The vector pCMV-lacZ was used as an internal control to measure the efficiency of each transfection. The reporter- and b-galactosidase-encoding vectors were used at 2.0 and 0.5 μg DNA per 60 mm dish, respectively, in calcium phosphate transfections and at 0.25 and 0.8 mg per 35mm dish, respectively, in liposome-mediated transfections.

The pCDNA3 plasmid (Invitrogen) contained the full length human wtERa cDNA to the amino terminus of which was added an eight amino acid FLAG epitope. This construct was used at 0.5 mg DNA per dish in calcium phosphate transfections and at 0.1 mg in liposome-mediated transfections. The serine to alanine phosphorylation site mutants were subcloned into the pCDNA3 expression vector.

The vectors pCDLSRa296 (39) and pCMV were used to express cyclin A, cyclin H, CDK7, CDK2, or the dominant negative mutant, CDK2TS. The pCMV5 plasmid expressed the cyclin-dependent kinase inhibitor, p27. Two micrograms of cyclin or CDK DNA was used for each 60 mm calcium phosphate transfection plate, except in Figure 7 where the amount of cyclin A-encoding plasmid was varied from 0.5 to 10.0 μg per dish. For liposome-mediated transfections, 0.3 -0.6 mg of cyclin or CDK expressing plasmids were used per dish.

Plasmid and cDNAs: Summary and Sources

Plasmid/cDNA	Source/Reference
pCDLSRa	Takebe, 1988
pCMV-lacZ (Suisse)	D. Picard
pCMV5-p27	Polyak, 1994
pCMV-hERb	J. Gustafsson
pCMV5 hER-S104A/S106A	B. Katzenellenbogen
XETL	D. Picard
pCDLSRa296-cyclin H	D. Morgan
pCDLSRa296-CDK7	D. Morgan
pCMV-hER-S118A	P. Chambon

Site-Directed Mutagenesis Using the Polymerase Chain Reaction and DNA Sequencing

Phosphorylation site mutants were generated via a two-step PCR process wherein overlapping PCR fragments (a "top" strand and a "bottom" strand) bearing the mutation of interest were mixed and amplified (23). The reactions were carried out on a Perkin Elmer GeneAmp 2400 System using Perkin Elmer reagents and Taq DNA Polymerase. Intermediate PCR products were separated from excess primer and template using the Quiagen PCR Purification Kit (Quiagen). This method was used to generate only the single phosphorylation site mutants. Double and triple phosphorylation site mutants were constructed by subcloning. All phosphorylation site mutants were sequenced to verify the existence of the desired base alterations and to guard against the inclusion of untoward mutations (Sequenase Version 2.0 DNA Sequencing Kit, USB).

Mammalian Cell Culture and Treatments

The cell lines used in these studies (HeLa, Hs 578Bst, U-2 OS, Sk-BR-3, and MCF7) were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium containing phenol red (DMEM, GIBCO/BRL) supplemented with 10% fetal

bovine serum (FBS, HyClone), 50 units/ml each of Penicillin and Streptomycin, (GIBCO/BRL) and 2 mM L-Glutamine (GIBCO/BRL). Transfections were performed in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS. SK-BR-3 cells were maintained in RPMI-1640 (GIBCO/BRL) containing phenol red supplemented with 10% fetal bovine serum (FBS, HyClone), 50 units/ml each of Penicillin and Streptomycin, (GIBCO/BRL) and 2 mM L-Glutamine (GIBCO/BRL). Transiently transfected of SK-BR-3 were cultured in phenol red-free RPMI-1640 supplemented with 0.5% stripped FBS.

Transient Transfections and Transcriptional Activity Assays

For transient transfections, cells were seeded into 60 mm dishes at 2 x 10^5 cells per dish and transfected by either the calcium phosphate or liposome (Lipofectamine®, GIBCO/BRL or Trans IT-100®, Mirus for MCF7, Figure 19) methods the following day as described (4). Following calcium phosphate transfection, cells were rinsed twice with PBS and re-fed with phenol red-free media supplemented with 10% charcoal-stripped FBS containing either 100 nM 17b-estradiol or the ethanol vehicle. Cells transfected using Lipofectamine® were incubated with the liposome-DNA mixture in serum-free media for three hours and re-fed with serum-containing media. CAT and b-galactosidase assays were performed 24 hours later as described (38). Luciferase assays were performed as described (9) and normalized to total cellular protein as measured via the Bradford Assay (BioRad).

Mammalian Protein Expression

Protein expression was monitored by preparing whole cell extracts. Cells were rinsed with PBS and scraped with a rubber policeman into a 15 ml conical, pelleted by centifugation and lysed for 10-15 minutes on ice in 30-60 µl of lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 1 mM EDTA, 1 mM DTT with protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM PMSF) and phosphatase inhibitors (1.0 mM NaF, 10 mM b-glycerophosphate, 1.0 mM sodium orthovanadate)]. Lysates were clarifed by centrifugation at 12,000 rpm at 4°C, for 10 minutes. The total protein concentration was measured using the Bradford Assay and equal amounts of protein were used to prepare whole cell extracts which were boiled with an equal volume of 2xSDS sample buffer, separated by 10-12.5% SDS-PAGE and transferred to Immobilon paper (Millipore).

GST-Protein Expression and Purification

Truncated versions of the human ERa cDNA coding for amino acids 1-82, 1-115 and amino acids 1-121 were cloned into the pGEX-4T-1 (Pharmacia Biotech). Phosphorylation site serine to threonine mutants were cloned into the ER 1-121 expressing plasmid. GST fusion proteins were expressed and purified as described (27).

Insect Culture and Baculovirus Methods

High Five^{TM} insect cells were maintained in Ex-Cell 405 Insect Culture Media (JRH Biosciences) at 27°C. Baculovirus vectors (10^{-7}pfus) engineered to express human cyclin A , an HA-tagged human CDK2, or a FLAG-tagged version of the full length ER were used separately or in combination to infect cells. The cyclin A, HA-CDK2, HA-CDK2, HA-CDK7 and cyclin H expressing baculoviruses were all kindly provided to us by D. Morgan. Cells (~$1x10^7$cells per 100 mm dish) were infected with 0.5 ml of virus in a final volume of 3.0 ml for two hours at 27°C and re-fed with 10 ml of Ex-Cell medium. Two days post-infection, cells were lysed on ice for 30 minutes in 0.5 ml of 120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT with protease and phosphatase inhibitors, as described above.

Immunoprecipitations

Insect cell immunoprecipitations were performed using approximately 100 µg of extract and 5 µg of the monoclonal antibody, 12CA5 (Boehringer Mannheim), directed against the CDK HA-epitope, 5 µg of a human cyclin A-specific polyclonal antibody (#06-138, Upstate Biotechnology) or 5 mg of a monoclonal antibody, M2 (Eastman Kodak), directed against the FLAG-epitope. Immune complexes were immobilized on Protein A/G agarose beads (Santa Cruz Biotechnology), washed 4 times in 0.5 ml of lysis buffer and used in *in vitro* kinase assays (cyclin A/CDK2) or Western blotted to test for protein-protein interactions (ER/cyclin A/CDK2).

Immunoblotting

Western blot analyses were performed according to standard methods. The antibodies used were as follows. A polyclonal antibody directed against the N-terminus of ER, ER-21, was kindly provided to us by Dr. Geoffrey Green. Cyclin A was visualized using the polyclonal antibody described above. A monoclonal antibody was used to detect CDK2 (# C18520, Transduction Labs). p27 expression was detected using a monoclonal

antibody (#K25020, Transduction Labs). Horseradish peroxidase (hrp)-coupled Protein A (KPL), or goat anti-mouse (or rabbit) -coupled hrp were used as secondary antibodies (Amersham). Alternatively, alkaline phosphatase (AP)-coupled goat anti-mouse or goat anti-rabbit antibodies were used (BioRad). The reactions were visualized using either the AP substrate, 5-bromo-4-chloro-indolylphosphate/Nitro Blue Tetrazolium (BCIP/NBT), or via enhanced chemiluminescence (ECL, Amersham).

In vitro Kinase Assays

The GST-ER substrate (10 μg), ER 1-82, ER 1-115 or ER 1-121, was absorbed to 100 μl of a 50% slurry of glutathione-sepharose® 4B beads (Pharmacia Biotech) for 20 minutes at room temperature and washed twice with DK buffer (50 mM potassium phosphate, pH 7.15, 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM DTT, 1 mM PMSF). The immobilized substrate was added to the immunopurified kinase subunit(s) and incubated on ice for 5 minutes prior to the initiation of the kinase reaction in a final volume of 150 μl, as described (27). The reaction products were separated by 12.5% SDS-PAGE, stained with Coomassie blue to visualize the receptor band, and autoradiography was performed from 5 to 30 minutes at room temperature. Aliquots of the reaction mixtures were also separated by SDS-PAGE and subjected to Western blot analysis to determine the levels of ER, cyclin A, and CDK2. An anti-phosphothreonine-specific monoclonal antibody (Biomeda) was used to detect threonine-phosphorylated ER.

In vivo Metabolic Labeling

HeLa cells (~$1x10^6$ cells per 100 mm dish) were transiently transfected with FLAG-ER and/or cyclin A using Lipofectamine® and metabolically labeled with 1 mCi/ml of ^{32}P-orthophosphate in 2 ml of phosphate-free DMEM for 2 hours at 37°C in the absence or presence of 100 nM 17b-estradiol. Cells were washed twice with PBS, placed on ice and lysed directly on the plate by adding 200 μl of high salt lysis buffer described previously. The *in vivo* labeled FLAG-tagged ER was immunopurified using 5 μg of the monoclonal anti-FLAG antibody, M2. The ER protein recovered by immunoprecipitation was resolved on 10% SDS-PAGE, silver-stained, dried and autoradiography was performed for 12 hours at room temperature to visualize the radiolabeled ER. The incorporated radioactivity was quantified using the NIH Image program to analyze the scanned autoradiogram and a digitalized version of the silver-stained gel.

7. CONCLUSIONS

ER-Dependent Transcriptional Activity is Enhanced by the Ectopic Expression of Cyclin A in Multiple Cell Lines

We have examined the effects of the cyclin A/CDK2 activation and inhibition on ER-dependent transcriptional enhancement. Here, we provide evidence that alterations in the regulation of cyclin A/CDK2 complex lead to changes in hormone-dependent and hormone-independent changes in ER transcriptional enhancement. Our findings indicate that the ectopic expression of cyclin A elevates ER transcriptional activity in a variety of cell types, including cervical adenocarcinoma cells (HeLa), osteosarcoma cells (U-2 OS), breast adenocarcinoma cells (SK-BR-3) and normal breast epithelial cells (Hs 578Bst) (Figure 4). Our results likely represent an underestimate of the full impact of cyclin A on ER transcriptional activity, since these findings were obtained in cells that contain endogenous cyclin A and CDK2. Since cell lines lacking these regulatory proteins have not been identified, studies aimed at investigating the effects of cyclin A/CDK2 complex activation necessarily rely upon overexpressing cyclin A above its background level.

Reciprocal Effects of Cyclin/CDK Activators and Inhibitors on ER-Dependent Transcriptional Enhancement

Consistent with the view that activation of the cyclin A/CDK2 complex increases ER transcriptional activity, is our observation that ER-dependent transcriptional activity is further enhanced by the concurrent expression of CAK but virtually abolished under conditions where cyclin A/CDK2 activity is suppressed by the kinase inhibitor p27, or by a dominant negative CDK2 mutant in HeLa cells (Figure 2). This demonstrates that the cyclin A/CDK2 effect upon ER transcriptional activation can be both increased and decreased. These observations first suggested to us that the level of CDK activation which is determined by a number of regulatory proteins ultimately governs ER transcriptional activity within a given cellular context.

Cyclin A/CDK2 Phosphorylation and Enhancement of ER-Dependent Transcriptional Activation Can be Mapped to S104, S106 and S118

Our findings also demonstrate that ER is a substrate for cyclin A/CDK2 kinase activity. ER is phosphorylated *in vitro* by cyclin A/CDK2 complexes and incorporation of phosphate into ER is stimulated by cyclin A expression *in vivo*. We have attempted to

further characterize the molecular effect of ectopic cyclin A expression and CDK2 activation upon the ER. Specifically, we have sought to map the ER N-terminal activation domain sites of cyclin A/CDK2 phosphorylation. Towards this end, we have utilized a variety of biochemical and genetic assays the combined results of which demonstrate that all three putative CDK phosphorylation sites, S104, S106 and S118 can be phosphorylated by the cyclin A/CDK2 complex *in vitro*. Furthermore, individual serine to alanine mutations at these sites decrease the effect of ectopic cyclin A expression *in vivo* to enhance ER-dependent transcriptional activation. The ER-dependent transcriptional activity of the double and triple serine to alanine mutants is further decreased compared to wtER under conditions of ectopic cyclin A expression.

Our results imply that an interdependence exists among the individual sites with regard to ER-transcriptional activation. However, additional studies will be necessary to fully characterize these relationships as they relate to receptor phosphorylation and transcriptional modulaion.

These findings serve to extend our understanding of how cell cycle regulatory proteins can impact upon transcription factors in general and upon ER in particular. Our approach of ectopically expressing a single CDK regulatory protein serves as a model whereby we can understand how upsetting the balance of cell cycle regulatory proteins can affect transcription factor function and, perhaps, contribute to inappropriate cellular proliferation.

Model for Cyclin A/CDK2 Regulation of ER-Dependent Transcriptional Activation

Figure 8 depicts a model for ER regulation by the cyclin A/CDK2 complex based on our findings. We propose that the cyclin A/CDK2 complex directly phosphorylates ER, and in doing so, facilitates its interaction with the basal transcriptional machinery or an ER coactivator, and thus, increases the receptor's ability to activate transcription. Inhibition of cyclin-dependent kinase activity by CKIs, such as p27, or through a reduction in cyclin or CDK expression, would decrease receptor phosphorylation, weakening these putative ER-transcription factor contacts, thus leading to decreased receptor transcriptional activity. We further envision that the expression of the CAK complex, cyclin H and CDK7, enhances ER transcriptional activation by increasing the activity of the endogenous cyclin A/CDK2 pool. Since cyclin H and CDK7 are also components of TFIIH (1, 14, 17, 36), we cannot exclude the possibility that this CAK complex may be acting at the level of TFIIH to increase its catalytic activity, which in turn, increases ER transcriptional activity. Together, these data suggest that the cyclin A/CDK2 kinase complex directly influences the ER's

transcriptional regulatory properties. We conclude that ultimately the *balance* of these CDK regulatory proteins determines kinase activity, which in this case translates into differential transcriptional activation by ER.

Conclusion and Physiological Significance for Breast Cancer

A complex picture of signal transduction by ER is emerging that appears to rely on the collaboration of multiple factors for its regulation, with each event in the pathway vulnerable to subversion. This subversion may take the form of aberrant expression of cyclin or CDK subunits, or cyclin-dependent kinase inhibitors, leading to an increase in receptor phosphorylation and activity which might contribute to uncontrolled cell proliferation. It has recently been recognized that cyclins, in particular cyclin D1, participate in normal breast development (37). This, together with a growing body of evidence linking cyclin overexpression and other mechanisms of CDK dysregulation to breast cancer, provides a direct connection between development and oncogenesis. Clearly, the involvement of cyclins, CDKs, CAKs and CKIs in ER-mediated transcriptional regulation is complex and will require further investigation.

The implications of our findings to the field of breast cancer diagnosis and treatment are promising. It has long been recognized that breast tumors are diverse in terms of genetic and molecular composition, proliferative and metastatic potential and response to hormone, radiation and chemotherapeutic interventions. Future directions of clinical investigation might include using the levels or relative ratios of CDK regulatory proteins to assess tumor stage, metastatic potential, or anticipated treatment outcomes. Since using p27 levels as a prognostic indicator for progression of lung, colon, and breast tumors has already been utilized with apparent success, this approach appears promising.

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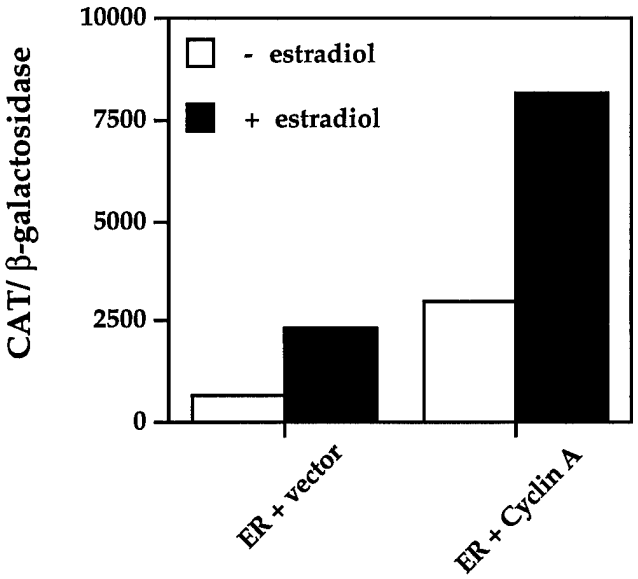
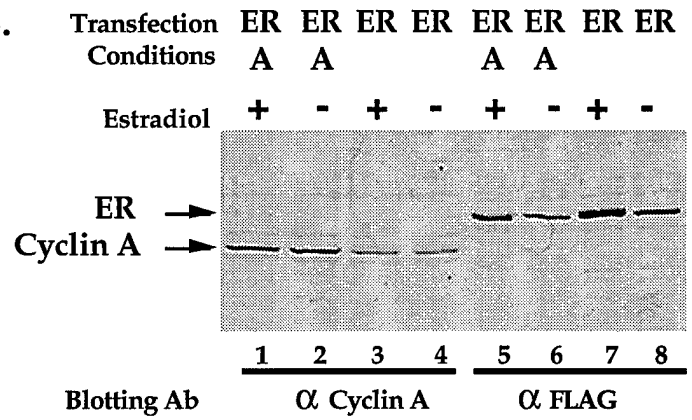
9. **APPENDICES** (Reprint, Figures and Figure Legends)

A.


10000
7500
5000
2500
0
CAT/ β-galactosidase
- estradiol
+ estradiol
ER + vector
ER + Cyclin A

B.


Transfection Conditions
ER ER ER ER ER ER ER ER
A A A A
Estradiol
+ - + - + - + -
ER
Cyclin A
1 2 3 4 5 6 7 8
Blotting Ab
α Cyclin A
α FLAG

C.

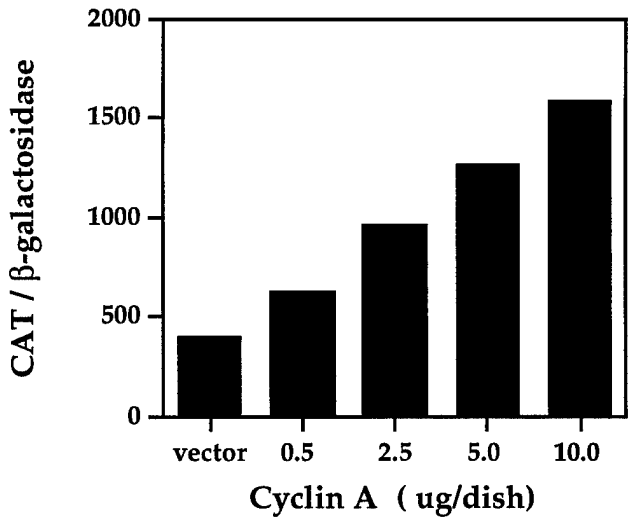

2000
1500
1000
500
0
CAT / β-galactosidase
vector
0.5
2.5
5.0
10.0
Cyclin A (ug/dish)

Figure 1. Activation of ER transcriptional enhancement by ectopic cyclin A expression. (A) ER-deficient HeLa cells (3 X 10^5 cells per 60 mm dish) were transiently transfected using Lipofectamine® with 2 μg of an ERE containing reporter plasmid, ΔETCO, possessing a single ERE upstream of a minimal promoter fused to the CAT gene, and 0.5 μg of the ER expression vector and 4 μg of the expression vector SRα296 (ER + vector) or 0.5 μg of the ER expression vector and 4 μg of SRα296-cyclin A (ER + cyclin A), along with 0.5 μg pCMV-lacZ as an internal standard for transfection efficiency. Cells were incubated with 100 nM 17β-estradiol or the ethanol vehicle as indicated for 24 hours, harvested and assayed for CAT and β-galactosidase activity. (B) Whole cell extracts were prepared from a parallel set of transfected cells as described in A. Equal amounts of protein (50 mg/ lane) were separated by 10% SDS-PAGE, transferred to Immobilon paper, probed with the M2 monoclonal antibody directed against the FLAG-epitope on ER or a polyclonal antibody against human cyclin A, and visualized with an alkaline phosphatase-conjugated goat secondary antibody. (C) Increasing amounts of cyclin A lead to increased ER transcriptional activity. Using the calcium phosphate procedure, HeLa cells were transiently transfected with increasing amounts of cyclin A (0.5 μg to 10.0 μg) with a constant amount of ER expression and reporter plasmids, and CAT activity was measured in the presence of 17β-estradiol. For transfection experiments, data represent the mean of at least two experiments done in duplicate whose error was less than 10%.

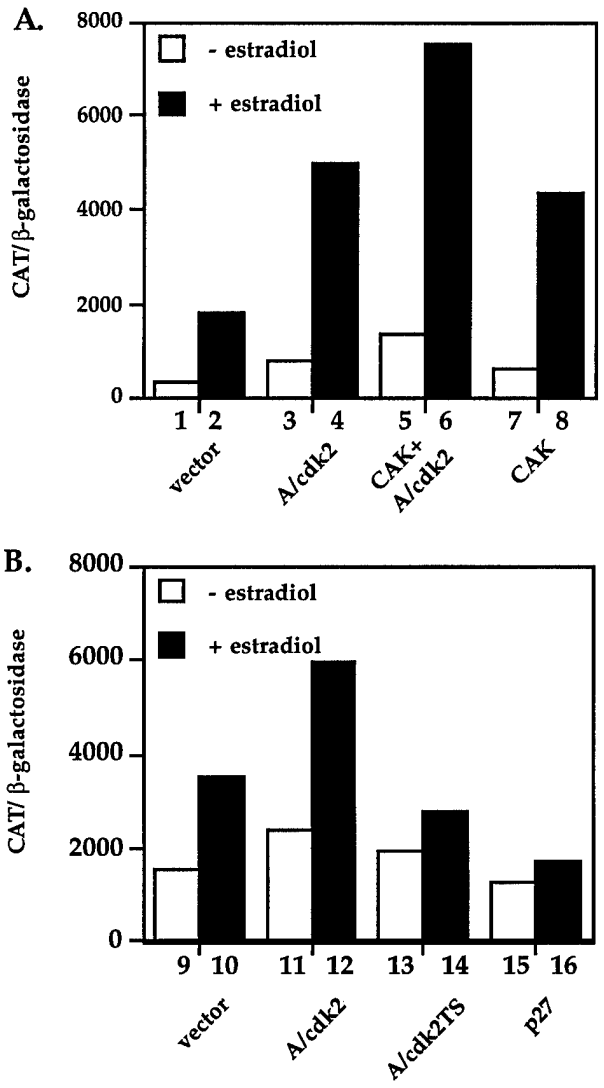


Figure 2. Effects of Cyclin/CDK Activators and Inhibitors on ER Transcriptional Enhancement. HeLa cells were transiently transfected using the calcium phosphate method with the reporter plasmid and a control empty expression vector (lanes 1-2 and 9-10); or expression vectors for cyclin A/CDK2 (lanes 3-4 and 11-12); cyclin A/CDK2 + CAK (lanes 5-6), CAK alone (lanes 7-8), cyclin A/CDK2TS (dominant negative) (lanes 13-14) and p27 (lanes 15-16). CAT activity was measured in the absence or presence of 100 nM 17β-estradiol.

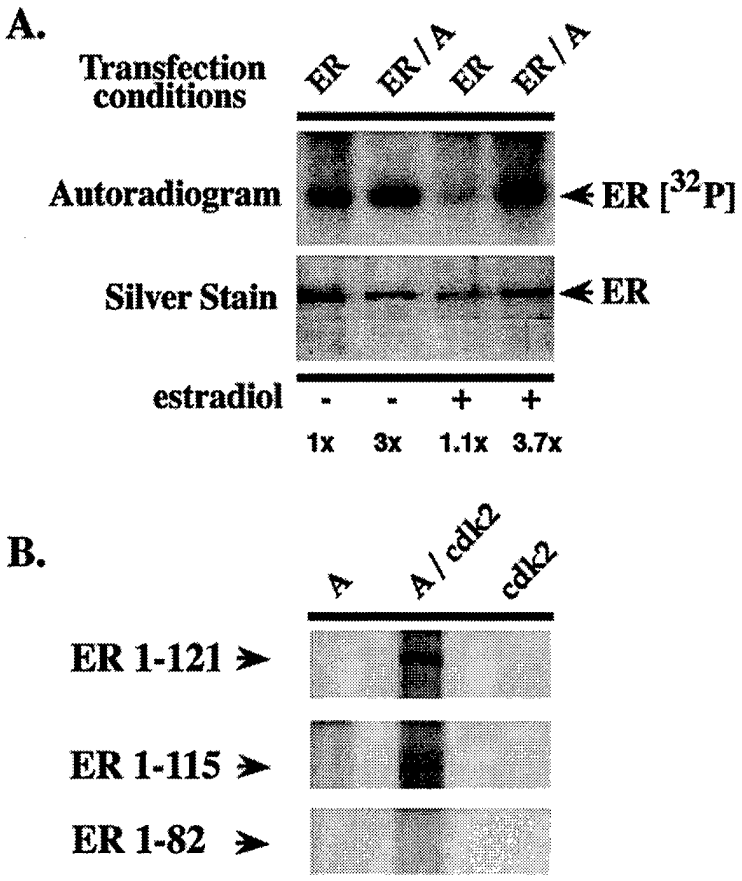


Figure 3. ER is Phosphorylated by the Cyclin A/CDK2 Complex. [A] Phosphorylation of ER *in vivo* in the absence and presence of cyclin A. HeLa cells were transfected with FLAG-ER in the absence and presence of cyclin A expression vector as described in Figure 1 and metabolically labeled with 1 mCi/ml of ^{32}P-orthophosphate for 2 hours at 37°C in the absence or presence of 100 nM 17β-estradiol. Whole cell extracts were prepared and ER was immunoprecipitated using the M2 monoclonal antibody. ER immunoprecipitates were separated by 10% SDS-PAGE, silver-stained to verify equal levels of ER in each lane (lower panel) and exposed to film to visualize the phosphorylated receptor (upper panel). The incorporated radioactivity was normalized to the amount of ER immunoprecipitated in each condition. The value of the untreated (-estradiol) ER was arbitrarily set as 1. [B] Phosphorylation of ER *in vitro* by the cyclin A/CDK2 kinase complex. Bacterially expressed GST-ER 1-82, 1-115 and 1-121 derivatives were absorbed onto glutathione agarose beads and used as substrates for *in vitro* kinase assays in the absence of estradiol. Cyclin A and CDK2 were produced in 5B insect cells, separately or in combination, purified by immunoprecipitation and used in the kinase assays. The reaction mixture was separated by 10% SDS-PAGE and the phosphorylated products were visualized by autoradiography.

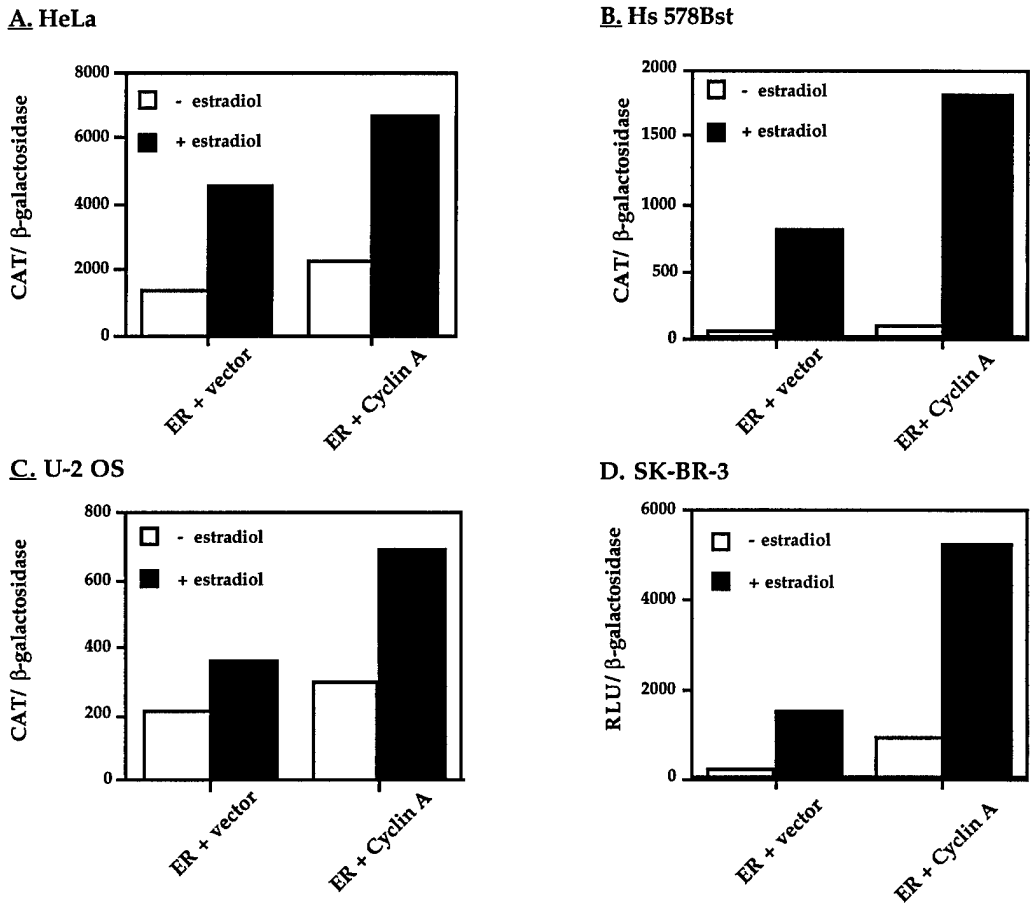


Figure 4. Cyclin A Enhances ER-Dependent Transcriptional Activation in Multiple Human Cell Lines. Four ER-negative human cell lines, [A] HeLa cells derived from a human cervical carcinoma, [B] Hs 578Bst, a human cell line derived from normal breast tissue, [C] U-2 OS, a human osteosarcoma cell line, and [D] SK-BR-3 cells, derived from a mammary adenocarcinoma pleural effusion, were transfected with ER expression and reporter plasmids. Cells were co-transfected with the empty expression vector or the expression vector encoding cyclin A. Cells were cultured in the presence and absence of 17β-estradiol and assayed for transcriptional activity as described.

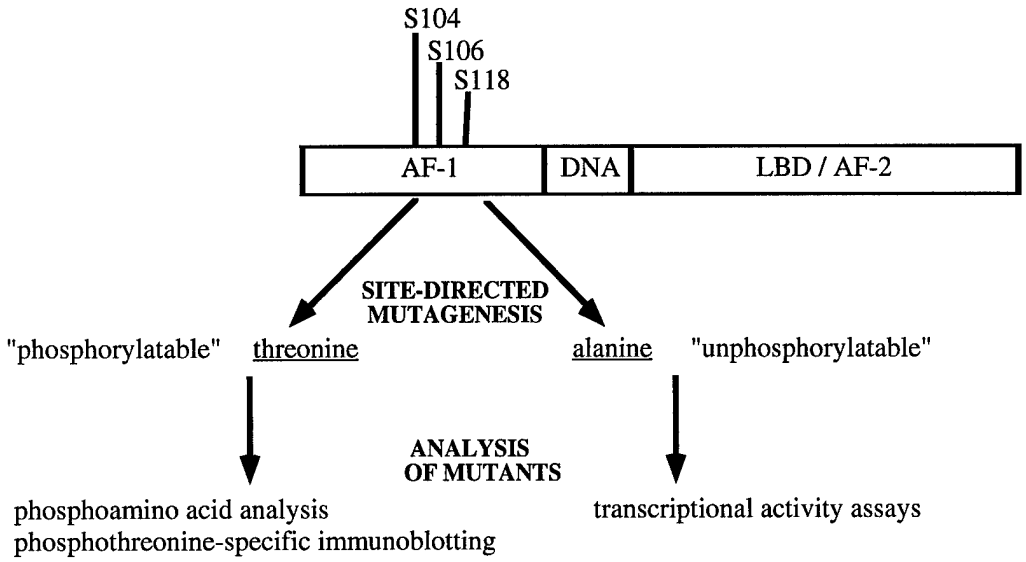


Figure 5. Strategy for Generating ER Phosphorylation Site-Directed Mutants. To identify which sites or sites are phosphorylated by the cyclin A/CDK2 complex, site-directed mutagenesis was performed to change S104, S106 and S118 to an unphosphorylatable residue, alanine or to a phosphorylatable threonine that can be both targeted by the cyclin A/CDK2 complex and discriminated from phosphoserine (see text). These serine to threonine and serine to alanine contructs can be used [1] To probe the effect of *in vitro* phosphorylation using the cyclin A/CDK2 complex upon the individual sites and [2] To assess the effect of mutations on ER-dependent transcriptional activity under conditions of ectopic cyclin A expression, respectively.

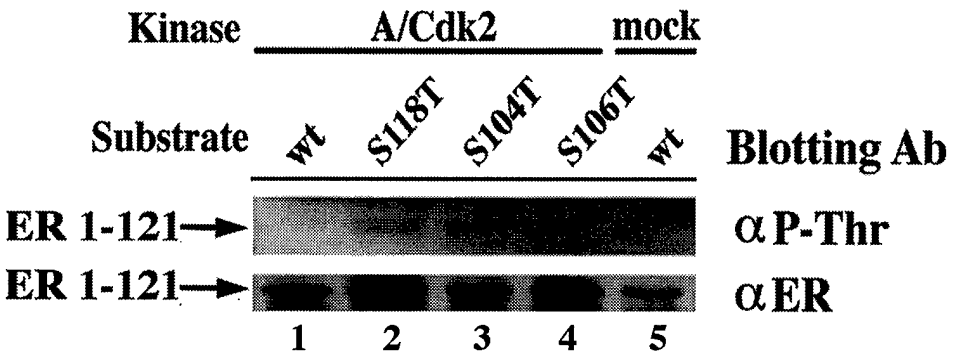


Figure 6. The Cyclin A/CDK2 Complex Phosphorylates Amino Acids 104, 106 and 118 *in vitro*: Phosphothreonine Detection by Western Blotting Using Serine to Threonine Swap Mutants. The wt and mutant ER 1-121 (S104T, S106T and S118T) protein derivatives were purified on glutathione beads and used as substrates for the cyclin A/CDK2 complex in a nonisotopic *in vitro* kinase assay (lanes 1-4). The reaction products were separated by 12.5% SDS-PAGE and transferred to Immobilon paper. Western blot analyses with a monclonal antibody specific for phosphothreonine [upper panel] and a polyclonal antibody specific for ER [lower panel] were performed The immunoblots were visualized with a Protein A-hrp-conjugated secondary antibody using ECL.

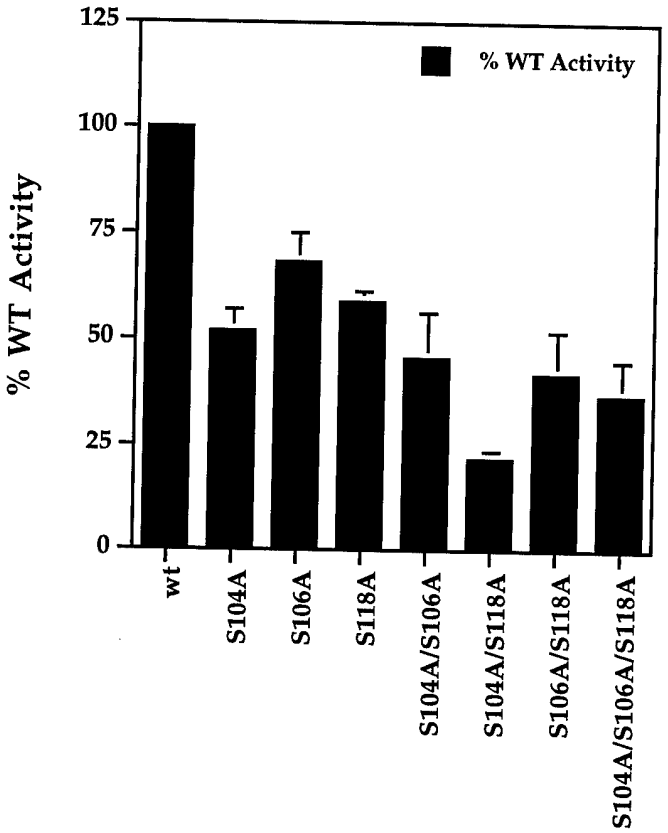


Figure 7. Summary of Phosphorylation Site-Directed Mutants: Average Percentage of Wild Type ER Cyclin A-Enhanced Activity.

The average percentage of wtER-dependent transcriptional activity in the presence of estradiol and under conditions of cyclin A co-transfection are shown. These data include a minimum of two assays (with two data points per assay) for each mutant construct. This is a graphical representation of the data summarized in Table 1.

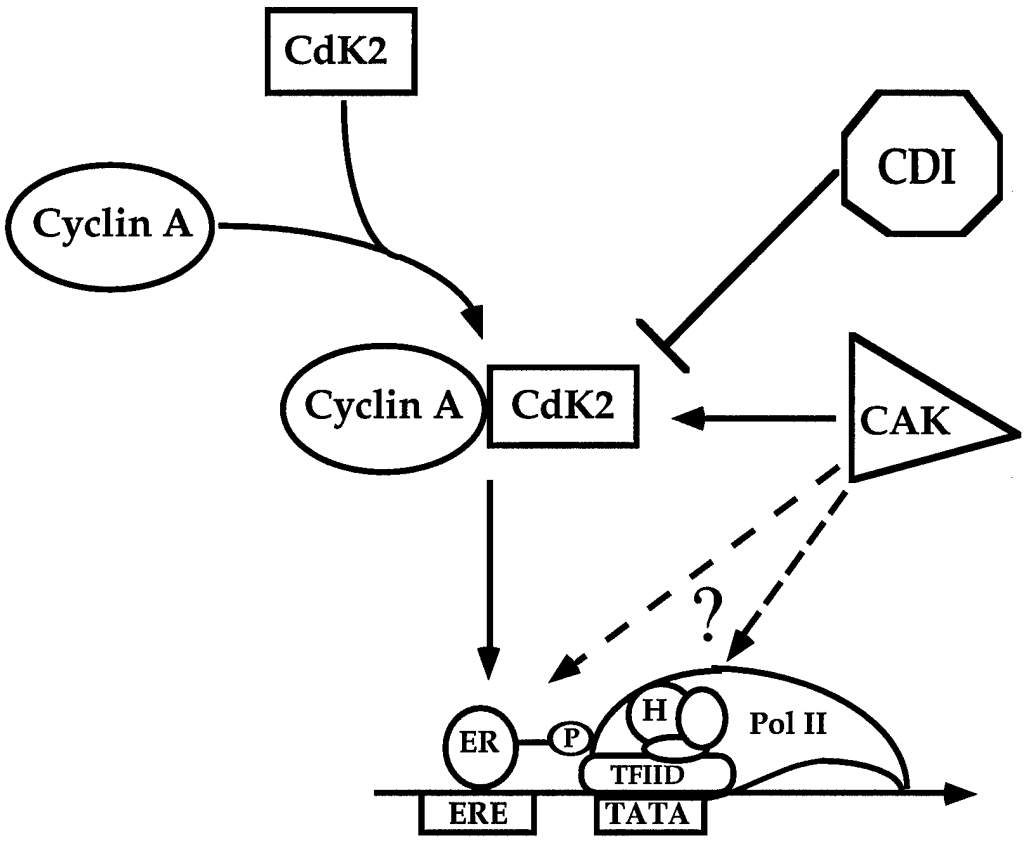


Figure 8. Model for ER Regulation by the Cyclin A/CDK2 Complex. The cyclin A/CDK2 complex phosphorylates ER which increases the receptor's ability to activate transcription by facilitating its interaction with the basal transcriptional machinery or an ER co-activator. Inhibiting cyclin-dependent kinase activity by CKIs would have the opposite effect, resulting in reduced ER phosphorylation and decreased receptor transcriptional activity. Expression of the CAK complex, cyclin H and CDK7, would enhance ER-dependent transcription by increasing the activity of the endogenous cyclin A/CDK2 pool. It is also conceivable that the CAK complex may be acting at the level of TFIIH to increase ER transcriptional enhancement. We conclude that it is the *balance* between the cyclins, CDKs and their regulatory proteins which will ultimately determine ER transcriptional activity.

Table 1. Summary of Effects of Phosphorylation Site Mutations Upon Cyclin A/ CDK2 Enhancement of ER Transcriptional Activity.

	S104A	S106A	S118A	S104A /S106A	S104A /S118A	S106A /S118A	S104A /S106A /S118A
%WT Activity	52 +/- 5	68 +/- 7	59 +/- 3	46 +/- 11	22 +/- 2	42 +/- 10	37 +/- 8
#Expt.	n=3	n=4	n=2	n=2	n=2	n=2	n=2
% Decrease in Activity	48	32	41	54	78	58	63

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Biochemistry

Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex

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ABSTRACT **We have found that ectopic expression of cyclin A increases hormone-dependent and hormone-independent transcriptional activation by the estrogen receptor *in vivo* in a number of cell lines, including HeLa cells, U-2 OS osteosarcoma cells and Hs 578Bst breast epithelial cells. This effect can be further enhanced in HeLa cells by the concurrent expression of the cyclin-dependent kinase activator, cyclin H, and cdk7, and abolished by expression of the cdk inhibitor, $p27^{KIP1}$, or by the expression of a dominant negative catalytically inactive cdk2 mutant. ER is phosphorylated between amino acids 82 and 121 *in vitro* by the cyclin A/cdk2 complex and incorporation of phosphate into ER is stimulated by ectopic expression of cyclin A *in vivo*. Together, these results strongly suggest a direct role for the cyclin A/cdk2 complex in phosphorylating ER and regulating its transcriptional activity.**

The estrogen receptor (ER) is a ligand-dependent transcriptional regulatory protein that controls the genetic programs affecting many aspects of cell growth and differentiation. In addition to ligand binding, phosphorylation plays an important role in regulating ER function. The receptor contains sites for both constitutive and ligand-dependent phosphorylation. Three serine residues (amino acids 104, 106, and 118) located within the N-terminal activation domain (AF-1) and one residue in the hinge region, S294, match the consensus sequence recognized by a family of serine/threonine proline-directed kinases that includes cyclin-dependent kinases (cdk), mitogen-activated protein kinases and glycogen synthase kinase-3. Ser-104, -106, and -118 are phosphorylated upon hormone treatment; serine to alanine mutations at these positions decrease ligand-dependent transcriptional activity (1–3). Accumulating evidence suggests that mitogen-activated protein kinase can phosphorylate Ser-118 and that this may lead to estradiol-independent ER activation or, alternatively, an increase in ligand-dependent ER activation. However, whether cdks target ER as a substrate for phosphorylation and affect its transcriptional activity remains unclear. Recently, a cdk-independent effect of cyclin D1 upon ER-dependent transcriptional activity was reported in T-47D breast cancer cells (4). A link between cdk enhancement of ER function and attendant receptor phosphorylation has not yet been demonstrated.

Cdks are a family of proteins composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. The cyclin subunit appears to regulate subcellular localization and timing of activation as well as substrate specificity of the kinase complex. Cdk complexes regulate the activity of target molecules, including transcriptional regulatory proteins, by phosphorylation. Regulation of cdk activity is accomplished by proteins that activate (cdk activators or CAKs), or inhibit (cdk inhibitors or CDIs), kinase function (5–8). Because cdks control cell division, the dysregulation of cyclins, their kinase partners, and/or the upstream regulatory CAKs and CDIs, have been implicated in the initiation and promotion of hyperplasia and oncogenesis. In fact, the overexpression of the regulatory cyclin subunit and the dysregulation of the catalytic cdk subunit have been identified in a number of solid tumors, leukemias, and tumor-derived cell lines (9–18).

This study examines the effects of the cyclin A/cdk2 complex on ER transcriptional activation. We chose to focus on cyclin A for several reasons: (*i*) Cyclin A plays a multifaceted role in cell cycle progression and is a key regulator of cdk2, a serine/threonine proline-directed kinase with the potential to phosphorylate ER. (*ii*) Cyclin A expression is cell adhesion-dependent, such that its overexpression can lead to adhesion-independent cell growth, a hallmark of cellular transformation (19, 20). (*iii*) The synthesis and degradation of cyclin A are tightly regulated, suggesting that its aberrant expression could seriously jeopardize the control of cell growth (21, 22). (*iv*) Cyclin A overexpression has been implicated as an important indicator of oncogenesis in several contexts including human breast tumor-derived cell lines and a mouse mammary tumor virus breast cancer model (11, 13, 23–25). (*v*) Cyclin A shares several features with the protooncogene cyclin D1 including the ability to bind to and phosphorylate the retinoblastoma protein, such that inappropriate cyclin A expression leads to perturbations in the regulation of the G1 to S transition (26–28). (*vi*) Recent reports have also linked the degradation of $p27^{KIP1}$ (hereafter referred to as p27), an inhibitor of cyclin A/cdk2 activity, and aggressive breast and colorectal cancers (15, 17, 18). Together, these findings suggest that cyclin A may function as a protooncogene. To determine whether the cyclin A/cdk2 complex can affect ER function, we have examined the consequences of activation or inhibition of the cyclin A/cdk2 pathway on ER-dependent transcriptional activation.

MATERIALS AND METHODS

Plasmids. A FLAG epitope was added to the N terminus of the full-length wild-type ER cDNA. This construct was inserted into the pCMV-Neo^r (Invitrogen) expression vector; 0.5 μg DNA per 60-mm dish was used in the transfections. The vector pCDLSRα296 was used to express cyclin A, cyclin H, cdk7, cdk2, or the dominant negative mutant, cdk2TS. The pCMV5 plasmid expressed p27. Two micrograms of cyclin or cdk DNA was used for each 60-mm transfection plate, except in Fig. 2*C* where the amount of cyclin A-encoding plasmid was varied from 0.5 to 10.0 μg per dish. The ΔETCO reporter plasmid contained one estrogen response element upstream of the thymidine kinase promoter (−109) driving the expression of the chloramphenicol acetyltransferase (CAT) gene. This

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Abbreviations: ER, estrogen receptor; cdk, cyclin-dependent kinase; CAK, cyclin-dependent kinase activator; CDI, cyclin-dependent kinase inhibitor; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; GST, glutathione *S*-transferase.

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reporter lacks a nearby activator protein-1 binding site to ensure that the results obtained are not influenced by other regulatory elements in the plasmid. The vector pCMV-lacZ was used as an internal control to measure the efficiency of each transfection. The reporter- and β-galactosidase (β-gal)-encoding vectors were used at 2.0 and 0.5 μg DNA per 60-mm dish, respectively.

Mammalian Cell Culture and Treatments. The cell lines used in these studies were obtained from the American Type Culture Collection and maintained in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone), 50 units/ml each of penicillin and streptomycin (GIBCO/BRL), and 2 mM L-glutamine (GIBCO/BRL). Transfections were performed in phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum. For transfections, cells were seeded into 60-mm dishes at 2×10^5 cells per dish and transfected the following day by either the calcium phosphate precipitation or the liposome-mediated (Lipofectamine, GIBCO/BRL) methods (29). At 12–16 h posttransfection, cells were rinsed twice with PBS and refed with phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum containing either 100 nM 17β-estradiol or the ethanol vehicle. CAT and β-gal assays were performed 24 h later as described (30). Protein expression was monitored by preparing whole cell extracts. Cells were lysed for 30 min on ice in 200 μl of high salt lysis buffer [400 mM NaCl/50 mM Tris·HCl, pH 8.0/0.5% Nonidet P-40/1 mM EDTA/1 mM DTT with protease inhibitors (1 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin A/1 mM phenylmethylsulfonyl fluoride)] and phosphatase inhibitors (1.0 mM NaF/10 mM β-glycerophosphate/1.0 mM sodium orthovanadate). Whole cell extract (100 μg) was separated by SDS/10% polyacrylamide gel and transferred to Immobilon paper (Millipore).

Glutathione *S*-Transferase (GST)-Protein Expression and Purification. Truncated versions of the human ER cDNA coding for amino acids 1–82, 1–115, and amino acids 1–121 were cloned into pGEX-5T-1 (Pharmacia). GST fusion proteins were expressed and purified as described (31).

Insect Cell Culture and Baculovirus Methods. High Five insect cells were maintained in Ex-Cell 405 Insect Culture Media (JRH Biosciences, Lenexa, KS) at 27°C. Baculovirus vectors (10^{-7} plaque-forming units) engineered to express human cyclin A or an hemagglutinin-tagged human cdk2 were used separately or in combination to infect cells. Cells (1×10^7cells per 100-mm dish) were infected with 0.5 ml of virus in a final volume of 3.0 ml for 2 h at 27°C and refed with 10 ml of Ex-Cell medium. Two days postinfection, cells were lysed on ice for 30 min in 0.5 ml of 120 mM NaCl, 50 mM Tris·HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT with protease and phosphatase inhibitors as described above.

Immunoprecipitations. Insect cell immunoprecipitations were performed using ≈100 μg of extract and 5 μg of the mAb 12CA5 (Boehringer Mannheim) directed against the cdk hemagglutinin-epitope, or 5 μg of a human cyclin A-specific polyclonal antibody (#06–138, Upstate Biotechnology, Lake Placid, NY). Immune complexes were immobilized on protein A/G agarose beads (Santa Cruz Biotechnology), washed four times in 0.5 ml of lysis buffer and used in the *in vitro* kinase assay.

***In Vitro* Kinase Assays.** The GST-ER substrate (10 μg), ER 1–82, ER 1–115, or ER 1–121, was absorbed to 100 μl of a 50% slurry of glutathione-Sepharose 4B beads (Pharmacia) for 20 min at room temperature and washed twice with kinase buffer (50 mM potassium phosphate, pH 7.15/10 mM $MgCl_2$/5 mM NaF/4.5 mM DTT/1 mM phenylmethylsulfonyl fluoride). The immobilized substrate was added to the immunopurified kinase subunit(s) and incubated on ice for 5 min prior to the initiation of the kinase reaction in a final volume of 150 μl as described (31). The reaction products were separated by 12.5% SDS/PAGE, stained with Coomassie blue to visualize the receptor band, and autoradiography was performed from 5 to 30 min at room temperature. Aliquots of the reaction mixtures were also separated by SDS/PAGE and subjected to Western blot analysis to determine the levels of ER, cyclin A, and cdk2.

***In Vivo* Metabolic Labeling.** HeLa cells (1×10^6 cells per 100-mm dish) were transiently transfected with FLAG-ER and/or cyclin A and metabolically labeled with 1 mCi/ml (1 Ci = 37 GBq) of [^{32}P]orthophosphate in 2 ml of phosphate-free DMEM for 2 h at 37°C in the absence or presence of 100 nM 17β-estradiol. Cells were washed twice with PBS, placed on ice, and lysed directly on the plate by adding 200 μl of high salt lysis buffer. The *in vivo* labeled FLAG-tagged ER was immunopurified using 5 μg of the monoclonal anti-FLAG antibody (M2, Eastman Kodak). The ER protein recovered by immunoprecipitation was resolved on SDS/10% polyacrylamide electrophoresis gel, silver-stained, and dried. Autoradiography was performed for 12 h at room temperature to visualize the radiolabeled ER. The incorporated radioactivity was quantified using the National Institutes of Health IMAGE program to analyze the scanned autoradiogram and a digitized version of the silver stained gel.

RESULTS

Increased ER Transcriptional Enhancement by Ectopic Cyclin A Expression. To establish whether ectopic expression of cyclin A affects ER-dependent activation, we examined the ability of cyclin A to increase ER-mediated transcriptional enhancement. ER-deficient HeLa cells were transfected with an expression vector for the full-length human ER containing a FLAG epitope at its N terminus, the reporter plasmid estrogen response element-thymidine kinase-CAT, plasmids encoding human cyclin A and a constitutive β-gal expression vector as an internal transfection standard. Transfected cells were treated with 17β-estradiol or the ethanol vehicle for 24 h. Transcriptional activity was measured by CAT assay and normalized to β-gal activity. As shown in Fig. 1*A*, both hormone-dependent and hormone-independent ER transcriptional activity were increased roughly 3-fold when cyclin A is overexpressed. No effect of cyclin A on reporter gene activity was observed in the absence of ER (not shown). To ensure that this increased transcriptional activity was not a result of additional ER protein production, we monitored protein expression in whole cell extracts using Western blot analysis. As Fig. 1*B* illustrates, ER levels are not increased by cyclin A coexpression (compare lanes 5 and 6 to lanes 7 and 8). In addition, cyclin A is expressed above endogenous levels as a result of our transient transfection scheme and estradiol treatment does not alter cyclin A expression (Fig. 1*B*, compare lanes 1 and 2 to lanes 3 and 4). By increasing the amount of cyclin A used in these transfections, we were able to observe a concomitant increase in ER transcriptional activation (Fig. 1*C*). Coexpression of cyclin A and cdk2 also results in an increased ER-dependent transcriptional activity slightly above that of cyclin A alone. Expression of cdk2 alone, on the other hand, did not significantly alter the ER-dependent transcriptional activity (not shown). These findings suggest that cyclin A is a limiting factor for full hormone-dependent ER-mediated transcriptional enhancement, presumably by favoring the formation of active cyclin/cdk complexes from endogenous cdk2 subunits. Thus, cyclin A expression greatly magnifies the characteristic hormone-dependent ER transcriptional response, which suggests that this cyclin/cdk complex can act as an effector of the ER signaling pathway.

Reciprocal Effects of cdk Activators and Inhibitors on ER Transcriptional Enhancement. To further demonstrate that alterations in cyclin A/cdk2 activity can modify ER transcriptional enhancement, we used two classes of cdk regulatory proteins, CAK, which is composed of cyclin H and cdk7, and the CDI, p27. p27 inhibits many cyclin/cdk complexes, includ-

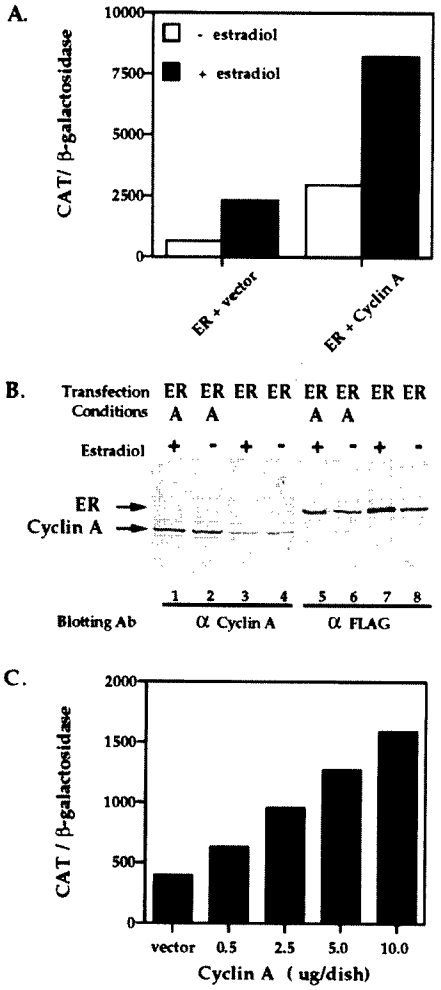

FIG. 1. Activation of ER transcriptional enhancement by ectopic cyclin A expression. (*A*) ER-deficient HeLa cells (2×10^5 cells per 60-mm dish) were transiently transfected using Lipofectamine with 2 μg of the estrogen response element containing reporter plasmid, possessing a single estrogen response element upstream of the thymidine kinase promoter fused to the CAT gene (ΔETCO), and 0.5 μg of the ER expression vector and 4 μg of the expression vector SRα296 (ER + vector) or 0.5 μg of the ER expression vector and 4 μg of SRα296-cyclin A (ER + cyclin A), along with 0.5 μg of pCMV-lacZ as an internal standard for transfection efficiency. Cells were incubated with 100 nM 17β-estradiol or the ethanol vehicle for 24 h as indicated, harvested and assayed for CAT and β-gal activity. (*B*) ER and cyclin A expression in transfected HeLa cells. Whole cell extracts were prepared from a parallel set of transfected cells. Equal amounts of protein (100 μg per lane) were separated by SDS/10% polyacrylamide gel, transferred to Immobilon paper, probed with the M2 monoclonal antibody directed against the FLAG-epitope on ER or a polyclonal antibody against human cyclin A, and visualized with an alkaline phosphatase-conjugated goat secondary antibody. (*C*) Increasing amounts of cyclin A lead to increased ER transcriptional activity. Using the calcium phosphate procedure, HeLa cells were transiently transfected with increasing amounts of cyclin A (0.5 μg to 10.0 μg) with a constant amount of ER expression and reporter plasmids, and CAT activity was measured in the presence of 17β-estradiol. For transfection experiments, data represent the mean of at least two experiments done in duplicate with <10% variation.

ing cyclin A/cdk2, cyclin E/cdk2, and cyclin D/cdk4 (32, 33). We were particularly interested in studying p27 in light of recent reports (15, 17, 18) linking its premature or excessive degradation to aggressive breast and colorectal cancers.

Fig. 2*A* illustrates that cdk activation by expression of cyclin A/cdk2 or CAK (cyclin H and cdk7) leads to a greater than 2-fold increase in both hormone-dependent and hormone-independent ER transcriptional activity. The coexpression of

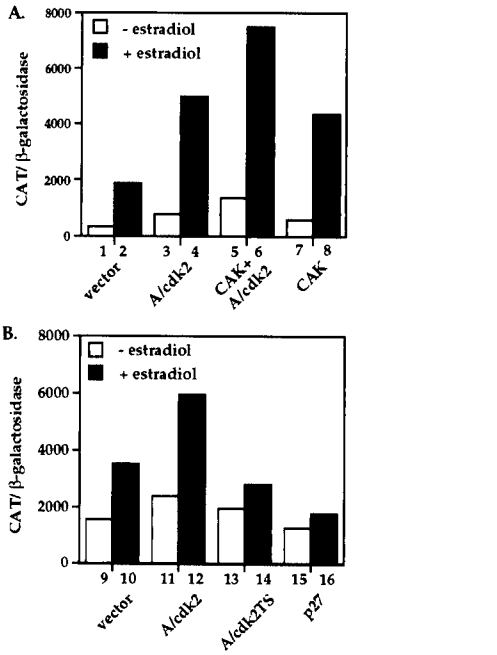

FIG. 2. Effects of cdk activators and inhibitors on ER transcriptional enhancement. (*A*) Effects of CAK (cyclin H/cdk7) on ER transcriptional activation. HeLa cells were transiently transfected using the calcium phosphate procedure with paired ER expression and reporter plasmids as described in Fig. 1, along with a control empty expression vector, (lanes 1 and 2); or expression vectors for cyclin A/cdk2 (lanes 3 and 4); cyclin A/cdk2 + CAK (lanes 5 and 6) and CAK alone (lanes 7 and 8). (*B*) Effects of cdk inhibitors on ER transcriptional activation. HeLa cells were transiently transfected with ER expression and reporter constructs along with an empty expression vector (lanes 9 and 10); or expression vectors for cyclin A/cdk2 (lanes 11 and 12); cyclin A/cdk2TS (dominant negative) (lanes 13 and 14) and p27 (lanes 15 and 16). Hormone treatment and activity assays were performed as described in Fig. 1. Data represent the mean of two experiments done in duplicate with <10% error.

all four proteins, the cyclin/cdk complex as well as the CAK complex, further augments (4-fold) this response and lends further support for cyclin/cdk involvement in the regulation of ER-dependent transcriptional activity.

We next asked if a decrease in cdk activity would reduce ER-dependent transcriptional activation. We chose two means of inhibiting cdk2 activity. Initially, the CDI, p27, was ectopically expressed in HeLa cells and ER-dependent transcriptional enhancement was measured. Ligand-dependent and, to a lesser degree, ligand-independent transcriptional activation by ER was reduced by p27 expression (Fig. 2*B*). This effect is noted in either the presence or absence of ectopically expressed cyclin A. Therefore, reducing cdk activity leads to impaired ER transcriptional activity.

At this point, we could not discriminate between an effect of p27 upon cdc2, cdk2, or cdk4, since p27 can inhibit all of these kinases. Therefore, we sought another means of reducing cdk2 activity by using a catalytically inactive cdk2 mutant to specifically block endogenous cdk2 activity. This cdk derivative, designated cdk2TS, is competent for cyclin A binding, but it cannot bind to ATP due to two consecutive amino acid changes in the ATP-binding site (Lys-33 and -34 are replaced by threonine and serine, respectively). This mutant acts as a dominant negative by sequestering cyclin A, thereby preventing it from binding and activating endogenous wild-type cdk2.

By expressing the dominant negative cdk2 mutant, we were able to reduce significantly the ER response to ligand treatment (Fig. 2*B*). Ectopic expression of a dominant negative cdc2 mutant had little effect on ER activity (not shown). These results strongly argue that the observed decrease of ER

transcriptional activity by p27 is due to inactivation of cdk2 and further suggests the importance of cyclin A/cdk2 enzymatic activity for hormone-dependent transcriptional enhancement by ER. It appears then, that the balance among the cdk regulatory proteins, cyclins, CAK, and CDIs, is critical in determining ER transcriptional activity.

Phosphorylation of ER by the Cyclin A/cdk2 Complex. Next, we investigated whether the cyclin A/cdk2 complex can phosphorylate ER. To determine if ectopic expression of cyclin A increased the amount of phosphate incorporated into ER *in vivo*, HeLa cells were transfected with ER alone or in combination with cyclin A and cells were metabolically labeled with [^{32}P]orthophosphate for 2 h in the presence or absence of 17β-estradiol. For each sample, the total amount of ER visualized by silver staining was used to standardize the amount of incorporated ^{32}P. The untreated ER condition was arbitrarily set as 1. As shown in Fig. 3*A*, ER phosphorylation is increased by ectopic expression of cyclin A in both the absence (3×) and presence (3.7×) of hormone. Thus, the presence of cyclin A increases incorporation of phosphate into ER by activating endogenous cdks.

To further investigate the effect of cyclin A/cdk2-dependent phosphorylation of ER we performed *in vitro* kinase assays. Three ER derivatives, containing amino acids 1–82, 1–115 or 1–121 were bacterially expressed and purified as GST-fusion proteins and used as substrates for phosphorylation by immunopurified baculovirus-expressed cyclin A and cdk2. These particular derivatives were chosen since the ER 1–121 derivative contains three serine-proline motifs at Ser-104, -106, and -118, whereas ER 1–115 contains only Ser-104 and -106. ER 1–82 lacks all of the putative serine-proline phosphorylation sites and thus serves as a negative control. Fig. 3*B* demonstrates that both ER 1–121 and ER 1–115 were phosphorylated by the cyclin A/cdk2 complex but not by either subunit alone. On the other hand, ER 1–82 was not phosphorylated by the cyclin A/cdk2 complex. In each reaction, expression of the ER substrate and the kinase subunits was verified by Western blotting and found to be identical (not shown). The fact that ER 1–121 and ER 1–115 derivatives were phosphorylated while ER 1–82 was not, strongly suggests that the residues contained in the region comprised by amino acids 83–121 comprise a motif targeted by the cyclin A/cdk2 complex. This data provide *in vitro* biochemical evidence that ER is a substrate for cyclin A/cdk2-dependent phosphorylation.

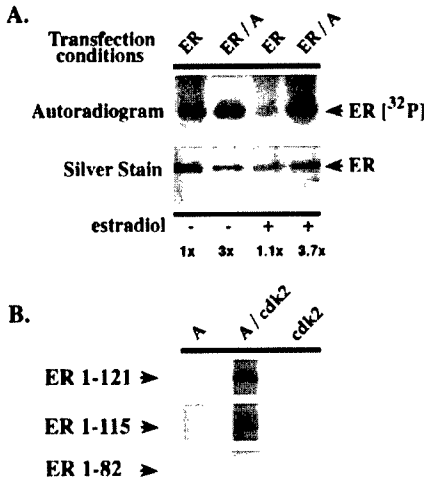

FIG. 3. ER phosphorylation by cyclin A/cdk2. (*A*) Phosphorylation of ER *in vivo* in the absence and presence of cyclin A. HeLa cells were transfected with FLAG-ER in the absence and presence of cyclin A expression vector using the Lipofectamine method as described in Fig. 1 and metabolically labeled with [^{32}P]orthophosphate as described in *Materials and Methods*. Whole cell extracts were prepared and ER was immunoprecipitated using the FLAG-mAb, M2. ER immunoprecipitates were separated by SDS/10% polyacrylamide gel, silver-stained (*Lower*), and exposed to film to visualize the phosphorylated receptor (*Upper*). The incorporated radioactivity was normalized to the amount of ER immunoprecipitated in each condition. The value of the untreated ER was arbitrarily set as 1. (*B*) Phosphorylation of ER *in vitro* by the cyclin A/cdk2 complex. Bacterially expressed GST ER 1–82, 1–115, and 1–121 derivatives were absorbed onto glutathione agarose beads and used as substrates for *in vitro* kinase assays. Cyclin A and cdk2 were produced in 5B insect cells, separately or in combination, purified by immunoprecipitation, and used in the kinase assays as described in *Materials and Methods*. The proteins were separated by SDS/10% polyacrylamide gel and the phosphorylated products were visualized by autoradiography.

Increased ER Transcriptional Enhancement in Response to Ectopic Cyclin A Expression in Multiple Cell Lines. To test our hypothesis that the regulation of ER-dependent transcriptional activity by the cyclin A/cdk2 complex is not specific to HeLa cells but rather reflects a general mode of regulation, we repeated our transcriptional activity assay in a variety of cell lines. We tested Hs 578Bst cells derived from breast tissue peripheral to an infiltrating ductal carcinoma and an ER-negative osteosarcoma cell line, U-2 OS. Cells were transiently transfected as described above, treated with 17β-estradiol or the ethanol vehicle for 24 h and transcriptional activity was measured. In the three cell types utilized, we observed a significant increase in ER ligand-dependent and -independent transcriptional activation (Fig. 4). These data imply that the ability of a cyclin A/cdk2 complex to enhance ER ligand-dependent transcription is conserved across multiple cell types.

DISCUSSION

We have examined the effects of cyclin A/cdk2 activation and inhibition on ER-dependent transcriptional enhancement. Here, we provide evidence that alterations in the regulation of the cyclin A/cdk2 complex lead to changes in both hormone-independent and hormone-dependent ER transcriptional enhancement. Our findings indicate that the ectopic expression of cyclin A elevates ER transcriptional activity and that this effect is not restricted to a single cell type. Our results likely represent an underestimate of the full impact of cyclin A on ER transcriptional activity, since these findings are obtained in cells that contain endogenous cyclin A and cdk2. Consistent with this view, ER transcriptional activity in both the presence and absence of hormone is virtually abolished under conditions where cyclin A/cdk2 activity is suppressed by the kinase inhibitor p27, or by a dominant negative cdk2 mutant. Thus it appears that cyclin A, the regulatory subunit of cdk2, is a limiting cofactor in the regulation of ER-dependent transcriptional activation in the cells examined.

Our findings also demonstrate that ER is a substrate for cyclin A/cdk2. ER is phosphorylated *in vitro* by cyclin A/cdk2 complexes and incorporation of phosphate into ER is stimulated by cyclin A expression *in vivo*. Importantly, our biochemical data demonstrate that the presence of three putative cdk target sites (Ser-104, Ser-106, Ser-118) is correlated with cyclin A/cdk2-dependent phosphorylation of the ER substrate *in vitro*. Work is ongoing to identify the precise residue(s) on the receptor phosphorylated by the cyclin A/cdk2 complex. Together, these data suggest that the cyclin A/cdk2 complex directly phosphorylates ER and that this modification serves to increase the receptor's transcriptional regulatory properties.

Zwijsen *et al.* (4) observed that overexpression of cyclin D1 increased ER-dependent transcriptional activation in T-47D cells without direct ER phosphorylation by cyclin D1/cdk4. Cyclin D1 appears to act indirectly as an ER cofactor, perhaps by tethering or phosphorylating other regulatory proteins that effect downstream signaling by ER. In contrast, we observed the cyclin A/cdk2 complex directly acting upon the receptor leading to its phosphorylation and a marked increase in

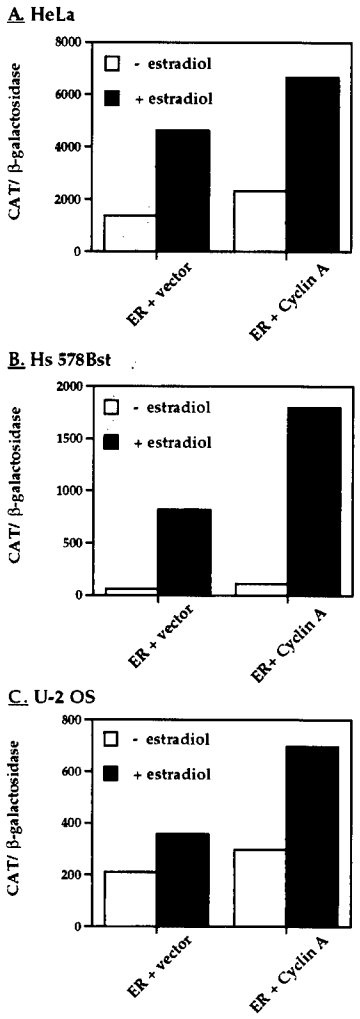

FIG. 4. Cyclin A enhances ER-dependent transcriptional activation in multiple human cell lines. Three ER-negative cell lines, (*A*) HeLa cells derived from a human cervical carcinoma, (*B*) Hs 578Bst, a human cell line derived from normal breast tissue, and (*C*) U-2 OS, a human osteosarcoma cell line, were transfected with ER expression and reporter plasmids as described in Fig. 1. Cells were also cotransfected with the empty expression vector or the expression vector encoding cyclin A. Hormone treatment and activity assays were performed as described in Fig. 1. Results shown represent a single experiment done in duplicate whose error was <10%. This experiment was repeated twice more with similar results.

ER-dependent transcriptional activity. Zwijsen *et al.* (4) did not observe an effect of cyclin A overexpression on ER-dependent transcriptional activation in T-47D cells and conversely, we failed to detect an increase in ER activity when cyclin D1 was ectopically expressed in either HeLa or ER-expressing U-2 OS human osteosarcoma cell line (J.M.T. and M.J.G., unpublished data). One factor contributing to the observed differences may lie in the cell types used in these studies. Recent reports and our own observations suggest that the level of expression of CDIs, such as p27, differ dramatically among cell types (34). Given that these proteins function as kinase inhibitors and as recently recognized cyclin D/cdk4 assembly factors, differences in CDI expression might significantly alter cdk signaling (34, 35). Among several breast cancer cell lines tested, T-47D cells were found to express high levels of p27 (34). This finding may account for the lack of a cyclin A effect in these cells, since the resulting cyclin A/cdk2 complex will be inhibited by endogenous p27. In contrast, HeLa cells used in this study express comparatively low amounts of p27, making ER-dependent transcription more sensitive to ectopic cyclin A expression. The cell-specific differences in the level of endogenous p27 may also help explain the ability of cyclin D1 to activate ER in T-47D cells, but not in HeLa cells, since abundant p27 may favor the formation of a cyclin D1/cdk4 complex, which may in turn phosphorylate an ER coactivator, or facilitate complex formation between ER and a receptor cofactor. Examination of the consequences of ectopic cyclin A expression in several breast cell lines has revealed an inverse correlation between cyclin A activation of ER transcriptional enhancement and the level of endogenous p27 (J.M.T. and M.J.G., unpublished data). Thus, the level of endogenous CDI may determine which cyclin isotypes will affect ER transcriptional activity, and may account for the observed differences between our findings and that of the Zwijsen *et al* (4).

Based on our findings, we propose a model for ER regulation by the cyclin A/cdk2 complex (Fig. 5). The cyclin A/cdk2 complex directly phosphorylates the receptor and in doing so, facilitates its interaction with the basal transcriptional machinery or an ER coactivator, which increases the receptor's ability to activate transcription. Inhibition of cdk activity by CDIs, such as p27, or through a reduction in cyclin or cdk expression, would decrease receptor phosphorylation, weakening these putative ER-transcription factor contacts, thus leading to decreased receptor transcriptional activity. We further envision that the expression of the CAK complex, cyclin H and cdk7, enhances ER transcriptional activation by increasing the activity of the endogenous cyclin A/cdk2 pool. Since cyclin H and cdk7 are also components of TFIIH (5, 36–38), we cannot exclude the possibility that this complex may be acting at the level of TFIIH to increase its catalytic activity, which in turn, increases ER transcriptional activity. Together, these data suggest that the cyclin A/cdk2 complex directly influences ER's transcriptional regulatory properties. We conclude that ultimately the balance of these cdk regulatory proteins determines kinase activity, which in this case translates into differential transcriptional activation by ER.

A complex picture of signal transduction by ER is emerging that appears to rely on the collaboration of multiple factors for its regulation, with each event in the pathway vulnerable to subversion. This subversion may take the form of aberrant expression of cyclin or cdk subunits, or CDIs, leading to alterations in receptor phosphorylation and activity that might contribute to uncontrolled cell proliferation. Clearly, the involvement of cyclins, cdks, CAKs, and CDIs in ER-mediated

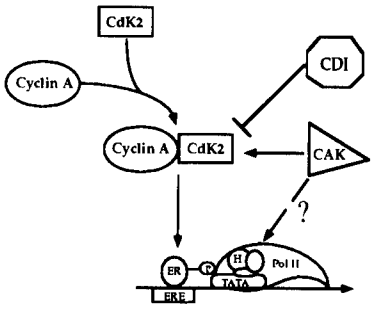

FIG. 5. Model for regulation of ER-dependent transcriptional activation by the cyclin A/cdk2 complex. The cyclin A/cdk2 complex phosphorylates ER which increases the receptor's ability to activate transcription by facilitating its interaction with the basal transcriptional machinery or an ER coactivator. Inhibiting cdk activity by CDIs has the opposite effect, resulting in reduced ER phosphorylation and decreased receptor transcriptional activity. Expression of the CAK complex, cyclin H, and cdk7, enhances ER-dependent transcription by increasing the activity of the endogenous cyclin A/cdk2 pool. It is also conceivable that the CAK complex may be acting at the level of TFIIH to increase ER transcriptional enhancement. We conclude that it is the balance among the cyclins, cdks, and their regulatory proteins that will ultimately determine ER transcriptional activity.

transcriptional regulation is complex and will require further investigation. It is likely, that phosphorylation events mediated by the cyclin/cdk pathway will emerge as a general mechanism of controlling steroid hormone action (31, 39).

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